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14. ABSTRACT

We have previously shown berberine, a natural compound, downregulates full-length androgen receptor (AR) and AR splice variants lacking the ligand-binding domain. During this grant period, we set out to investigate the underlying mechanisms. We found that berberine reduced full-length AR by inhibiting AR transcription, and inducing AR poly-ubiquitination, leading to protein degradation via the proteasome pathway. In contrast, the suppressive action on AR splice variants was exerted mainly through its inhibition of AR transcription, as berberine did not influence the degradation of the splice variants. We also found that these variants were intrinsically unstable, having a half-life much shorter than that of full-length AR. This could lead to a rapid depletion of the splice variants when protein synthesis is halted by berberine due to its reduction of AR transcripts. Additionally, we tested the efficacy of berberine in a Pten knockout model. We found that berberine inhibited tumor growth, but had no adverse effects on normal organs. Since Pten loss or inactivation is common in prostate cancer, these results provide support for the use of berberine in the prevention and treatment of prostate cancer.

15. SUBJECT TERMS
Prostate cancer; Androgen receptor; Berberine; Pten knockout

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Introduction

We have previously shown that berberine (BBR), a plant-derived isoquinoline alkaloid, suppresses androgen receptor (AR) signaling in prostate cancer cells (1). Interesting, we found that AR splice variants appear to be more susceptible to BBR downregulation than the full-length AR. During the first grant period, we set out to understand the mechanisms of BBR-induced downregulation of different AR isoforms (Task 1). We have also found that BBR inhibits the AKT pathway in prostate cancer cells. In this period, we have successfully generated Pten knockout mice and evaluated the efficiency of BBR in this animal model (Task 2).

Body

Task 1. To investigate the mechanisms of berberine-induced downregulation of full-length and splice variants of AR.

Berberine inhibits the transcription of the AR gene. We have previous concluded that BBR does not affect the mRNA level of full-length AR (1). The conclusion was drawn when β-actin was used as the housekeeping gene. However, we have since found that β-actin is ill-suited for this purpose since its transcript level was affected by BBR (data not shown). We decided to re-examine and compare the effects of BBR on the transcript levels of full-length AR and AR-V7/AR3. We chose ribosome protein L30 (RPL30), which has been identified as one of the most stably expressed gene (2), to normalize the gene expression data in this analysis. 22Rv1 cells were treated with 100 µM BBR and for up to 24 h and transcript levels of full-length AR and AR-V7/AR3 were analyzed by qRT-PCR. As shown in Fig. 1, both AR and AR-V7 mRNAs were reduced by BBR, starting at the 4 h time point. The suppression became more significant as a function of time. Interestingly, the change of AR-V7/AR3 mRNA paralleled that of AR in this experiment. This observation is supported by a recent publication by Liu et al, showing that AR-V7 transcript levels were highly correlated with full-length AR levels (3). The authors concluded that the splicing of AR-V7 RNA is coupled to the transcription rate of the AR gene. Based on these results, we conclude that BBR inhibits AR transcription and thereby decreases full-length AR and AR-V7/AR3 to similar magnitudes.

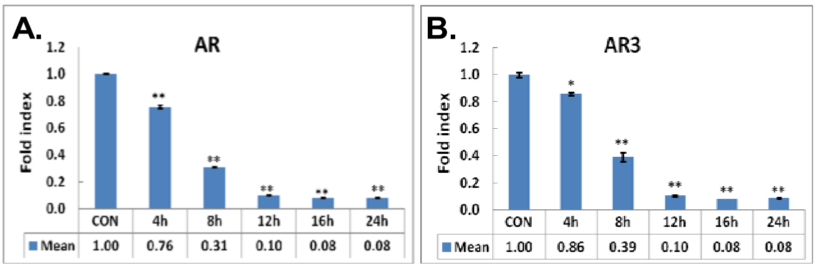
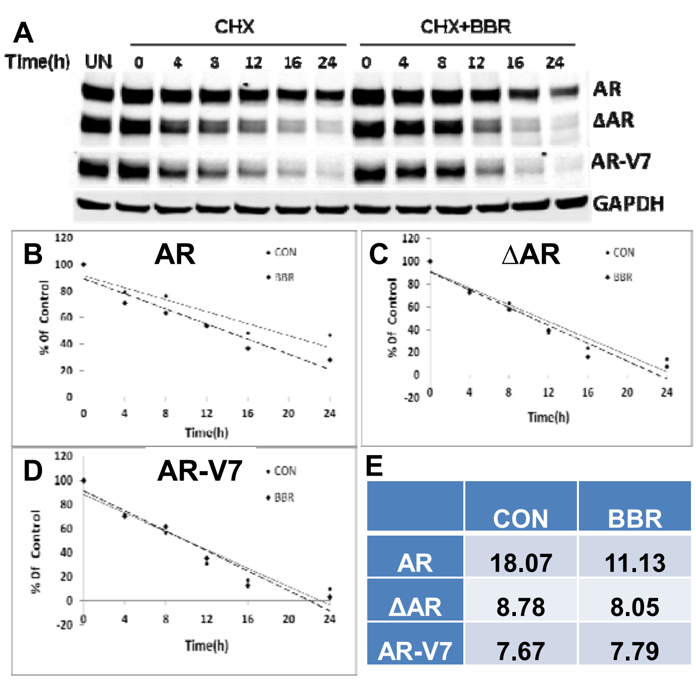


Fig. 1. BBR decreases mRNA levels of full-length AR (A) and AR-V7/AR3 (B). 22Rv1 cells were cultured in RPMI 1640 supplemented with 10% FBS and treated with 100 µM BBR for various durations. The transcripts were measured by qRT-PCR, with RPL30 as the housekeeping gene. The results were averaged from three independent experiments, and presented as mean ± standard deviation (SD). *, P<0.05; **, P<0.01.

BBR does not induce the degradation of AR splice variants. We have previously shown that BBR decreases the half-life of full-length AR (1). To determine if BBR exerts a similar effect on the splice variants, 22Rv1 cells were pretreated with cycloheximide to stop

protein synthesis and then treated with BBR. Cells were lysed at different intervals for Western blotting analysis, using antibodies recognize all isoforms (AR and ΔAR) or AR-V7 only. Normalized AR protein levels were analyzed by linear regression to determine the half-life. The results are shown in Fig. 2. The half-life of full-length AR was estimated to be ~18 hr, and it was decreased by BBR treatment (Fig. 2 B&E). This is consistent with our previous data from LNCaP cells. However, the splice variants (ΔAR) were much less stable than AR, with a much shorter half-life than that of AR (Fig. 2 C&E). Surprisingly, BBR treatment had little effect on the degradation of the splice variants. These results were confirmed by using an antibody specific for AR-V7/AR3 (Fig. 2 D &E). Similar results were obtained from LN95 cells (data not shown).



	CON	BBR
AR	18.07	11.13
ΔAR	8.78	8.05
AR-V7	7.67	7.79

Fig. 2. Berberine does not induce the degradation of AR splice variants. 22Rv1 cells were treated with 50 μg/mL cycloheximide (CHX) for 30 minutes and then with 100 μM BBR. **A**, Western analysis of cell lysates obtained at different intervals. **B-D**, normalized AR protein levels were analyzed by linear regression. **E**, half-life (hr) of AR proteins calculated from B-D.

BBR induces AR ploy-ubiquitinatin. To determine the effect of BBR on AR poly-ubiquitination, we performed an *in vivo* ubiquitination assay. As shown in Fig. 3, BBR markedly increased the intensity of ubiquitinated-AR in the presence of the proteasome inhibitor MG-132. This result suggested that BBR-induced AR degradation is mediated through the ubiquitin-proteasome pathway.

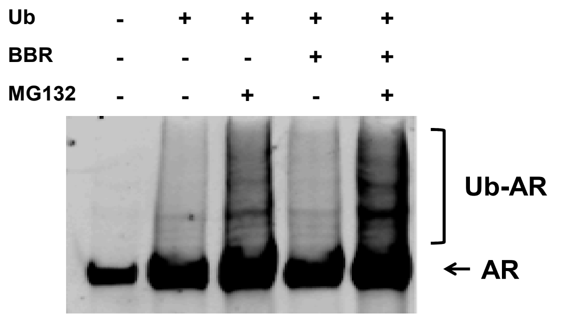


Fig 3. BBR induces AR poly-ubiquitination. LNCaP cells were transfected with plasmids encoding AR and his-tagged ubiquitin. Forty-eight hours after transfection, cells were treated with 100 μM BBR for 8h, then with 10 μM MG132 for 4 h. Following His-tag pulldown using Ni-NTA magnetic agarose beads, ubiquitinated proteins were detected by Western blot using an anti-AR antibody.

♂ PB-Cre4^{+} X ♀ *Pten*$^{loxP/loxP}$

↓

F1: ♂ *Pten*$^{loxP/+}$; Cre4^{+} X ♀ *Pten*$^{loxP/loxP}$

↓

F2: ♂ ***Pten*$^{loxP/loxP}$; Cre4^{+}** ⟶ ***Pten*$^{-/-}$**

♂ ***Pten*$^{loxP/loxP}$; Cre4^{-}** ⟶ ***Pten*$^{+/+}$**

♂ *Pten*$^{+/loxP}$; Cre4^{+}

♂ *Pten*$^{+/loxP}$; Cre4^{-}

Fig. 4. Breeding scheme to generate *Pten* knockout and wild-type mice. Genotyping was done by PCR using Cre- and Pten-specific primers.

Task 2. To evaluate the in vivo efficacy of berberine against prostate cancer growth in Pten knockout mice.

BBR inhibits prostate cancer growth in Pten knockout mice. Conditional Pten-KO mice were generated in-house by crossing PTEN$^{loxP/loxP}$ mice with PB-Cre4 mice, wherein the Cre recombinase is under the control of a modified prostate-specific probasin promoter (see Breeding scheme, Fig. 4). Genotyping was performed by PCR using primers specific for Pten and Cre. Male F2 mice (Pten-/- and Pten+/+) were randomly assigned to control or BBR treatment group (n=6) at the age of 12 weeks and received vehicle (DMSO) or 5 mg/kg/day of BBR, respectively, through i.p. injection. The treatment continued for 9 weeks and body weight of the mice was measured weekly. At the end of experiment, animals were sacrificed by CO_2 inhalation. The genitourinary bloc (GU bloc), consisting of the prostate lobes, seminal vesicles, ampullary glands, bladder, proximal ductus deferens, and proximal urethra was excised *en bloc*. The weight of the GU bloc is proportional to the prostate weight, thus it is widely used to represent prostate tumor burden (4–6). As shown in Fig. 5, Pten knockout mice have a markedly higher GU bloc weight than the wild-type mice, suggesting the presence of prostate tumors. In Pten knockout mice, treatment with BBR significantly reduced the weight of GU blocs (P<0.001). In contrast, there is no difference between treatment and control groups in wild-type mice. These results show that BBR inhibits prostate cancer growth without affecting the normal prostate. This result is consistent with our observation from the xenograft study (1), suggesting that the inhibitory action of BBR may be specific to the malignant tissue. We are currently developing an assay to measure BBR levels in the normal and malignant prostatic tissues to determine whether BBR is accumulated at a higher level in the malignant tissue.

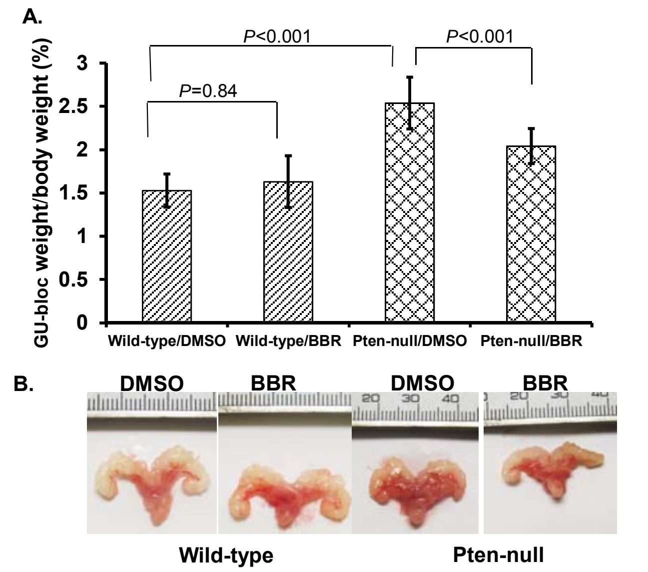


Fig. 5. Berberine treatment decreased prostate cancer growth in Pten knockout mice. A. The weight of the GU-bloc is normalized by body weight and presented as mean±standard deviation, n=6. **B.** Representative photos of the GU bloc in each group.

BBR has low toxicity in mice. The dose of BBR used in this study appears to be well tolerated; we did not observe any signs of stress in mice from the treatment groups. When body weight was compared between treatment and control groups, no difference was detected in both genotypes (Fig. 6 A&B). In addition, no difference was found in the weights of various organs, including liver, heart, lung, spleen, kidney, and testis among different groups (Fig. 6C). These results suggest BBR has a low toxicity profile. Further histological evaluation of the tissues is under way.

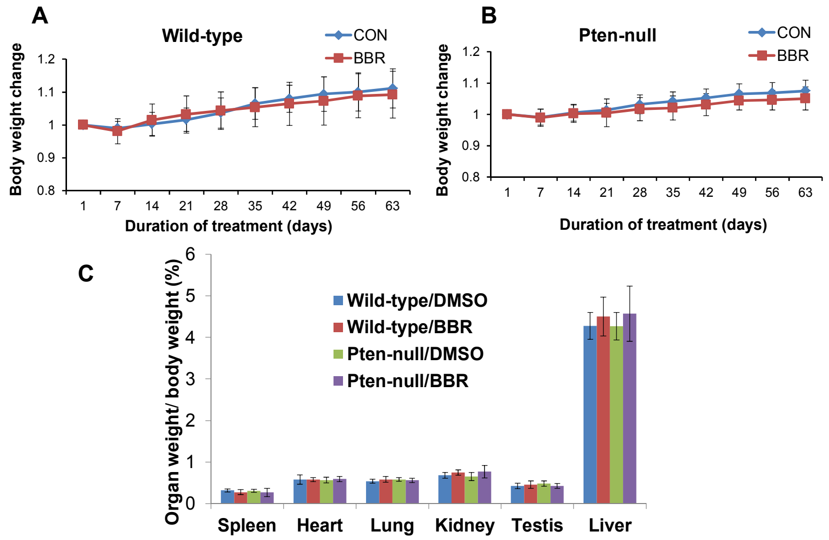


Fig. 6. Toxicity evaluation of berberine. A and **B**, change of body weight during the course of the experiment in wild-type and Pten-null mice, respectively. Body weight is expressed as fold of initial body weight. **C.** weight of various organs normalized by body weight and presented as mean± SD.

Immunohistochemistry (IHC) analysis. IHC staining for detecting AR, AKT, Ki-67, as well as TUNEL assay for apoptosis detection are currently under way. Due to the labor intensive nature of these assays, we have not completed the assays at this point.

Key Research Accomplishments

- We have established that BBR downregulates full-length AR by inhibiting AR transcription and inducing AR degradation via the ubiquitin-proteasome pathway.
- BBR inhibits AR splice variants mainly through inhibiting AR transcription, which in turn affects alternative splicing.
- AR splice variants are much less stable than full-length AR.
- We have demonstrated BBR inhibits prostate cancer growth driven by activated PI3K/AKT.

Reportable Outcomes

Using preliminary data generated with the support of this grant, we have further developed the project into an application and submitted it to the Research Scholar Grant at the American Cancer Society. The proposal, titled “Co-targeting AR and AKT by Berberine in Castration-resistant Prostate Cancer”, was reviewed in March, 2013 and received an OUTSTANDING rating. The proposal was approved by ACS for funding. However, due to fund

shortage, it is given a "Pay-if" designation and put on the waiting list. The proposal is payable once funds become available and remains eligible until June 30, 2015.

Conclusion

Through the experiments conducted in Task 1, we have reached the following conclusions: 1) BBR inhibits the transcription of the AR gene. This is supported by the observation that BBR induced a similar decline of full-length AR and AR-V7/AR3 across all the time points, and by a recent publication showing that splicing of AR variants is coupled with AR transcription. 2). BBR induces degradation of full-length AR via the ubiquitin-proteasome pathway. 3). BBR does not affect the degradation of AR splice variants. Recently, it has been shown ligand-binding domain contains sites for that the sites for AR ubiquitination (7). Therefore, it is possible that BBR induces AR ubiquitination via the sites in the ligand-binding domain. Truncation of the ligand-binding domain in the splice variants removes the sites for BBR-induced ubiquitination. 4) AR splice variants are intrinsically less stable than the full-length AR, as shown by the shorter half-life. Therefore, our previous observation that BBR is more efficient in downregulating the splice variants could not be attributed to increased protein degradation. Instead, BBR dramatically decreases the AR mRNAs (by 90% at 12 h), leading to much reduced synthesis of all AR proteins (full-length and splice variants). Since the splice variants are less stable, it is likely that they are depleted more rapidly than the full-length protein under BBR treatment.

In Task 2, we concluded that BBR is effective in blocking prostate cancer growth in a Pten conditional knockout model.

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